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Characterization of the *cycHJKL* Genes Involved in Cytochrome *c* Biogenesis and Symbiotic Nitrogen Fixation in *Rhizobium leguminosarum*

MARIA-JESUS DELGADO,[1] KAY H. YEOMAN,[2] GUANGHUI WU,[3] CARMEN VARGAS,[1]†
ANDREA E. DAVIES,[1] ROBERT K. POOLE,[3] ANDREW W. B. JOHNSTON,[2]
AND J. ALLAN DOWNIE[1]*

John Innes Centre, Norwich NR4 7UH,[1] University of East Anglia, Norwich NR4 7TJ,[2] and King's College London, London W8 7AH,[3] United Kingdom

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Mutants of *Rhizobium leguminosarum* bv. *viciae* unable to respire via the cytochrome aa_3 pathway were identified by the inability to oxidize *N,N′*-dimethyl-*p*-phenylenediamine. Two mutants which were complemented by cosmid pIJ1942 from an *R. leguminosarum* clone bank were identified. Although pea nodules induced by these mutants contained many bacteroids, no symbiotic nitrogen fixation was detected. Heme staining of cellular proteins revealed that all cytochrome *c*-type heme proteins were absent. These mutants lacked spectroscopically detectable cytochrome *c*, but cytochromes aa_3 and *d* were present, the latter at a higher-than-normal level. DNA sequence analysis of complementing plasmids revealed four apparently cotranscribed open reading frames (*cycH*, *cycJ*, *cycK*, and *cycL*). CycH, CycJ, CycK, and CycL are homologous to *Bradyrhizobium japonicum* and *Rhizobium meliloti* proteins thought to be involved in the attachment of heme to cytochrome *c* apoproteins; CycK and CycL are also homologous to the *Rhodobacter capsulatus ccl1* and *ccl2* gene products and the *Escherichia coli nrfE* and *nrfF* gene products involved in the assembly of *c*-type cytochromes. The absence of cytochrome *c* heme proteins in these *R. leguminosarum* mutants is consistent with the view that the *cycHJKL* operon could be involved in the attachment of heme to apocytochrome *c*.

Within legume nodules, leghemoglobin is an oxygen carrier that maintains a low free oxygen concentration at an optimal level (3 to 30 nM) for nitrogen fixation by rhizobia (1). During nodule development, these bacteria differentiate into bacteroids, which acquire the ability to fix nitrogen. It is now evident that one aspect of bacteroid differentiation is the development of a specialized branch of the respiratory pathway terminated by an oxidase with a very high affinity for oxygen (16, 22). Analyses of rhizobial mutants affected in both respiration and nitrogen fixation have helped define the components of the respiratory pathway required for normal nitrogen fixation. Strains of *Bradyrhizobium japonicum* mutated in the *fbcFH* genes encoding the cytochrome bc_1 complex cannot fix nitrogen, indicating that in nodules, bacteroids respire via a branch of the respiratory chain that includes cytochromes bc_1 and is distinct from the pathways terminated by cytochromes *d* and/or *o* (36). The *fixNOQP* operon encodes components essential for respiration during symbiotic nitrogen fixation. The product of *fixN* was predicted to be a heme *b*- and copper-binding subunit of an oxidase, while *fixO* and *fixP* were predicted to encode *c*-type cytochromes of about 28 and 32 kDa, respectively (16, 22). The predicted *fixQ* gene product is a small protein (M_r, 6,031) that is likely to be an integral membrane protein like FixN, FixO, and FixP (16, 22).

Given the predicted participation of three *c*-type cytochromes (c_1, FixO, and FixP) in bacteroid respiration, the proteins involved in biogenesis of *c*-type cytochromes should also be necessary for nitrogen fixation. Analyses of cytochrome *c*-deficient mutants of *Rhodobacter capsulatus* led to the identification of the *helABCDX* gene cluster (2, 3), and there are very similar genes in *B. japonicum* (23, 24) and *Rhizobium leguminosarum* (37). Some of the genes in these clusters are probably involved in translocating heme across the bacterial inner membrane, while other gene products (HelX and its homologs), which are related to thioredoxin, may be involved in the reduction of cysteines in apocytochrome *c* before heme attachment (2, 24, 35, 37). Mutations in these rhizobial genes block symbiotic nitrogen fixation, although several of these mutants can grow aerobically (24, 35, 37), presumably by relying on the cytochrome *d*- and/or cytochrome *o*-terminated branches of the electron transport pathways, which do not involve *c*-type cytochromes.

Two *Rhodobacter capsulatus* genes, *ccl1* and *ccl2*, are thought to be involved in the attachment of heme to cytochrome *c* apoproteins (3). Recent results (12, 26, 27) indicate that homologs of *ccl1* and *ccl2* are encoded by the last two genes in a four-gene operon, *cycHJKL*, that has been identified in *B. japonicum* and *Rhizobium meliloti*. Here we have identified a similar operon in *R. leguminosarum* and shown that it is necessary for cytochrome *c* biogenesis and symbiotic nitrogen fixation.

MATERIALS AND METHODS

Microbiological techniques. The bacterial strains and plasmids used are described in Table 1, Fig. 1, and below. TY complete medium (4) and Y minimal medium (31) containing succinate (10 mM) or mannitol (0.5% [wt/vol]) and glutamate (10 mM) were used for the growth of *R. leguminosarum*. *Escherichia coli* was grown in L medium (28). Antibiotics were added at the concentrations described previously (37). Tn*5* mutagenesis and isolation of respiration-deficient mutants were done as described previously (37) by screening for mutants with the NADI cytochrome oxidase test (17), staining Tn*5*-mutagenized colonies on TY medium, and screening for colonies that did not stain blue. Plants were grown and inoculated with *R. leguminosarum* strains as described previously (13).

Molecular genetic techniques. The library of *R. leguminosarum* DNA sub-

* Corresponding author. Mailing address: John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom. Phone: (1603) 452571. Fax: (1603) 456844. Electronic mail address: downie@bbsrc.ac.uk.

† Present address: Departamento Microbiologia y Parasitologia, Facultad de Farmacia, Universidad de Sevilla, 41012 Seville, Spain.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference
Strains		
8401	*R. leguminosarum* cured of a symbiotic plasmid; Str^r	15
A33	Rifampin-resistant derivative of 8401 carrying the *R. leguminosarum* bv. *viciae* symbiotic plasmid pRL1JI; previously called 8400/pRL1JI	8
A266	Derivative of A33 carrying *cycK67*::Tn*5*	This work
A267	Derivative of A33 carrying *cycH271*::Tn*5*	This work
A329	Derivative of 8401 carrying *cycK67*::Tn*5*	This work
A334	Derivative of 8401 carrying *cycH271*::Tn*5*	This work
Plasmids		
pML123	Broad-host-range cloning vector; Gen^r	14
pIJ1942	Cosmid carrying ≈30 kb of *R. leguminosarum* DNA, including *cycHJKL*	This work
pIJ1978	Derivative of pML123 carrying *cycHJ*	This work
pIJ7076	Derivative of pML123 carrying *cycHJKL*	This work
pIJ7091	Derivative of pML123 carrying *cycJKL*	This work
pIJ7097	Derivative of pUC18 carrying *cycK67*::Tn*5* on an *Eco*RI fragment	This work
pIJ7108	Derivative of pIJ7097 carrying part of Tn*5* plus flanking DNA	This work
pIJ7110	Derivative of pIJ7097 carrying the part of Tn*5* and flanking DNA not present in pIJ7108	This work
pIJ7115	Derivative of pUC18 carrying part of Tn*5* and the DNA flanking the *cycH271*::Tn*5* allele	This work

cloned in pLAFR1 (8, 10) was transferred by conjugation into the $NADI^-$ mutant A329 by using the helper plasmid pRK2013 (10) in a triparental filter mating and selecting for transconjugants on TY medium containing streptomycin and tetracycline. The transconjugants were screened with NADI reagent to identify blue ($NADI^+$) colonies. Plasmid DNAs were isolated from several such colonies and used to transform *E. coli* to tetracycline resistance. One such plasmid, pIJ1942, was confirmed to complement A329 to $NADI^+$ after conjugal transfer from *E. coli*. This plasmid was then transferred into a series of 30 other $NADI^-$ mutants; only one (A334) was complemented by pIJ1942.

*Hin*dIII-, *Sac*I-, or *Bam*HI-digested DNA from pIJ1942 was ligated with DNA of the broad-host-range vector pML123 digested with the same enzymes. Each ligation mix was used to transform *E. coli* to gentamicin resistance, and each set of transformed colonies was pooled. These three pools were separately transferred by conjugation into A329 and A334, selecting for resistance to gentamicin and streptomycin on TY medium. The six sets of transconjugants were screened for $NADI^+$ colonies, and DNA preparations were isolated from complemented transconjugants. Only those plasmids containing single DNA inserts were chosen for further work. A derivative of pML123 carrying a 10-kb *Hin*dIII fragment which complemented both mutants was called pIJ7076. A plasmid (pIJ7091) carrying a 5.0-kb *Bam*HI fragment complemented A329, but no plasmid carrying a single *Bam*HI fragment complemented A334. Plasmid pIJ1978 carrying a 3.3-kb *Sac*I fragment complemented A334, but no plasmid carrying a *Sac*I fragment complemented A329 (Fig. 1).

The *Eco*RI fragment carrying the Tn*5* insertion from A329 was cloned by digesting genomic DNA with *Eco*RI, ligating it with *Eco*RI-digested pUC18 (28), and transforming *E. coli*, selecting for resistance to ampicillin and kanamycin. pIJ7097 carries an 8.2-kb *Eco*RI fragment from A329 corresponding to a 2.5-kb *Eco*RI fragment containing Tn*5* (5.7 kb). pIJ7108 was derived from pIJ7097 after *Bam*HI digestion and elimination of a 4.5-kb *Bam*HI fragment containing part of Tn*5*. pIJ7110 contains the 4.5-kb *Bam*HI fragment from pIJ7097 cloned in Bluescript SK^+ (Stratagene). Similarly, DNA flanking the Tn*5* insertion in A334 was cloned as a 3.7-kb *Bam*HI fragment in pUC18 to form pIJ7115, selecting for kanamycin-resistant transformants (Fig. 1).

The DNA sequences adjacent to the ends of the Tn*5* insertions were obtained by sequencing pIJ7108, pIJ7110, and pIJ7115 with a 25-nucleotide primer (37) homologous to the inverted repeat sequence of Tn*5* between nucleotides 55 and 30 from the ends.

DNA sequence analysis. The DNA sequence presented here was derived from a series of restriction fragments subcloned from pIJ7076 into Bluescript SK^+. Nested deletions were generated by using exonuclease III and the Promega Erase-a-base kit. The DNA sequence was generated by the dideoxy chain termination method with a combination of double-stranded sequencing on an automated Pharmacia ALF sequencer (following the manufacturer's instructions) and single-stranded sequencing with 5′-[α-^{33}P]dATP and the U.S. Biochemical Corp. sequence kit, with 7-deaza-dGTP used to minimize sequence ambiguities. The sequence covering the open reading frames (ORFs) indicated (Fig. 1) was deduced from data obtained from both strands by using overlapping sequences.

Manipulation of DNA. Restriction enzyme digests, DNA hybridization, and DNA preparations were all carried out by standard procedures (28). Genomic *R. leguminosarum* DNA digested with appropriate enzymes was loaded at 5 μg per track and transferred to Hybond N (Amersham) for hybridization experiments. Radioactive probes were prepared from plasmids or DNA fragments excised from gels by using the Pharmacia Ready-to-Go DNA labelling kit according to the manufacturer's instructions.

Cell fractionation, protein gel electrophoresis, and heme staining. *R. leguminosarum* cells from a 300-ml culture grown in Y-succinate medium were harvested and resuspended in 3 ml of 100 mM potassium-phosphate buffer (pH 7.0) containing 100 μM 4-amidinophenylmethanesulfonyl fluoride and 20 μg of DNase I ml^{-1}. Cells were disrupted by using a French pressure cell (SLM Aminco). The cell extract was centrifuged at 20,000 × *g* for 20 min to remove unbroken cells, and the supernatant was then centrifuged at 140,000 × *g* for 2 h. The membrane pellet was resuspended in 2 ml of the same buffer, centrifuged at 140,000 × *g* for 2 h, and then resuspended in 100 μl of buffer. The supernatant soluble fraction from the first centrifugation at 140,000 × *g* was concentrated with a Centricon-10 filter. Protein concentrations were estimated by the method of Bradford (6), with bovine serum albumin as the standard. Membrane and soluble fractions were suspended in loading buffer (124 mM Tris [pH 7.0], 20% glycerol, 4.6% sodium dodecyl sulfate [SDS]) and electrophoresed on an SDS–12% polyacrylamide gel at room temperature. Proteins were transferred to a nitrocellulose filter and stained for heme-dependent peroxidase activity by chemiluminescence as described previously (37).

Measurements of spectra. Spectra were determined with a Johnson Foundation dual-wavelength SDB3 spectrophotometer. For spectra, bacteria were cultured in 500 ml of Y modified medium (K_2HPO_4, 0.022%; KH_2PO_4, 0.011%; $MgSO_4 \cdot 7H_2O$, 0.01%; $CaCl_2 \cdot 6H_2O$, 0.022%) containing 1% trace element solution (21) vitamins, sodium glutamate, and mannitol or succinate as the carbon source. After 3 days of growth at 30°C, cells were harvested, washed once with 25 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (pH 7.2), and then suspended in 5 ml of the same buffer.

Cells were reduced with a few grains of dithionite or oxidized with a few grains of ammonium persulfate. For the reduced-plus-CO minus reduced spectrum, dithionite-reduced cells were bubbled for 1 min with CO. For the photodissociation spectrum, dithionite-reduced cells in 30% (vol/vol) ethylene glycol were bubbled with CO for 1 min, cooled to −20°C for 5 min, and then cooled to −78°C for 10 min in the dark before equilibration at −100°C in the sample compartment of a Johnson Foundation SDB3 spectrophotometer. The sample was scanned twice to generate a baseline (reduced-plus-CO spectrum minus reduced-plus-CO spectrum) and then photolysed for 1 min by using a focused 200-W light beam. Each spectrum shown is the difference between the CO-dissociated sample and the reduced-plus-CO sample.

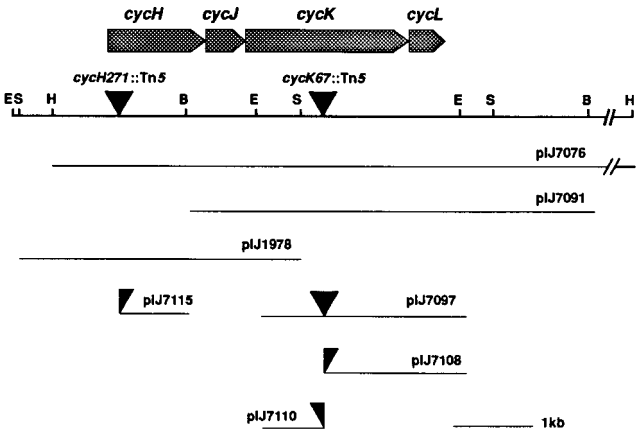

FIG. 1. Map of the *cycHJKL* gene region. The thick line represents part of the DNA region cloned in pIJ1942 and shows the positions of the relevant restriction sites of *Eco*RI (E), *Sac*I (S), *Hin*dIII (H), and *Bam*HI (B). The locations of Tn*5* insertions in mutants A267 (*cycH271*::Tn*5*) and A266 (*cycK67*::Tn*5*) are indicated by triangles. The cloned DNA fragments of various plasmids are also indicated. The triangle in pIJ7097 represents the entirety of Tn*5*, and the half triangles in pIJ7115, pIJ7108, and pIJ7110 represent subcloned parts of Tn*5*. The locations of the predicted gene products are shown as cross-hatched arrows.

Nucleotide sequence accession number. The nucleotide sequence of the *cycHJKL* operon of *R. leguminosarum* has been deposited in EMBL under accession number X89726.

RESULTS

Isolation of respiration-defective Fix$^-$ mutants. Mutants of *R. leguminosarum* 8401 with altered respiration were identified by screening a population of Tn*5*-mutagenized colonies for the inability to oxidize *N*,*N*′-dimethyl-*p*-phenylenediamine and α-naphthol (NADI reagent) to form an indophenol blue compound. Among the mutants identified, one (A329) was complemented by pIJ1942; it was identified by mating a cosmid library of *R. leguminosarum* DNA into A329 and screening for tetracycline-resistant colonies that oxidized the NADI reagent. When pIJ1942 was crossed into the other NADI$^-$ mutants that had been isolated, one (A334) was complemented to NADI$^+$ by this plasmid. Genomic DNAs from these two mutants were separately digested with *Bam*HI or *Sac*I and probed with pIJ1942 and the 3.5-kb internal *Hin*dIII fragment from Tn*5*. The patterns of hybridizing bands revealed that the mutations in each mutant were due to different single Tn*5* insertions in the region of DNA homologous to pIJ1942. The sizes of the hybridizing fragments obtained with A329 were consistent with Tn*5* insertion into a 5-kb *Bam*HI fragment and a 2.3-kb *Sac*I fragment. With mutant A334, Tn*5* was inserted into a 4.0-kb *Bam*HI fragment and a 3.3-kb *Sac*I fragment.

The Tn*5* insertions in A329 and A334 were transferred to *R. leguminosarum* A33 by transduction (7) with phage RL38 and selection for the kanamycin resistance specified by Tn*5*; the transductants, A266 and A267, respectively, were NADI$^-$. DNA hybridizations of *Bam*HI- and *Sac*I-digested genomic DNAs with pIJ1942 confirmed that they had the same patterns of fragments as did A329 and A334, respectively; pIJ1942 complemented both transductants to NADI$^+$. When they were inoculated onto pea seedlings, both A266 and A267 formed nodules, but the nodules did not fix nitrogen, as judged by the poor growth of the plants, the green color of the nodules, and the complete inability of the nodulated root systems to reduce acetylene to ethylene. When derivatives of A266 and A267 carrying pIJ1942 were inoculated onto plants, the nodules reduced acetylene. The levels of acetylene reduction by individual nodules varied, ranging from 20 to 80% of normal. However, the level correlated with the percentage of bacteria that retained the complementing plasmid pIJ1942, confirming that this plasmid complemented both the NADI$^-$ and the symbiotic nitrogen fixation phenotypes.

Light microscopy of sections of young pea nodules formed by A266 and A267 revealed identical phenotypes; there were many infected plant cells and many bacteroids in each cell (Fig. 2). In contrast, several respiration-deficient mutants of *B. japonicum* and *Rhizobium tropici* appear to induce nodules with few infected cells, each of which contained relatively few bacteroids (26, 32). Infected cells in the mature zones of the pea nodules induced by both A266 and A267 were surrounded by starch granules, a characteristic of nodule cells that contain bacteria unable to fix nitrogen.

Respiratory mutants A266 and A267 lack *c*-type cytochromes. Reduced minus oxidized difference spectra of intact cells of mutants A266 (Fig. 3b and c) and A267 (not shown) failed to reveal significant levels of *c*-type cytochromes compared with the spectrum of the wild-type control (Fig. 3a). The α peak at 550 nm attributed (37) to cytochrome *c* in the control strain was absent. This region of the spectra of these mutants was dominated by a signal at 560 nm attributed to cytochrome(s) *b*. In the Soret region, the peak of absorption of the mutants was smaller and at a higher wavelength (432 nm) than that of the wild type (426 nm), reflecting the absence of absorption due to cytochrome *c*. In cells grown for 3 days in mannitol medium, signals due to cytochrome *d* (reduced form at 630 nm and oxygenated form at 650 nm) were unusually prominent (Fig. 3b). The signal at 595 nm is presumably due to the high-spin cytochrome b_{595} component of the cytochrome *bd*-type oxidase complex, as it is in the well-studied *E. coli* and *Azotobacter vinelandii* oxidases (20). This signal tends to obscure the presence of cytochrome aa_3. However, for cells from cultures grown with succinate (rather than mannitol) as the carbon source, the cytochrome *bd* signals were weaker and a peak at 602 nm was clearly visible, indicating that cytochrome aa_3 can be made by these mutants (Fig. 3c). CO difference spectra were recorded to identify CO-binding heme proteins and putative terminal oxidases in mannitol-grown cells. The presence of cytochrome *d* was confirmed by a prominent signal at 640 nm due to carbon-monoxy-cytochrome *d* (Fig. 3d) and the loss of A_{620}. The undulations between 530 and 570 nm, the 416-nm peak, and the 430-nm shoulder are all tentatively attributed to cytochrome *o*, although more definitive data are required to demonstrate its oxidase function. The prominent trough at 440 nm may contain contributions from either cytochrome a_3 or the cytochrome b_{595} component of the cytochrome *bd*-type oxidase discussed above. The peaks and troughs of the Soret photodissociation spectrum obtained with mannitol-grown cells (Fig. 3e) and recorded at low temperature (photodissociated, i.e., reduced minus CO reduced) indicate that there is little cytochrome a_3 present, although the shoulders at 430 and 446 nm may indicate a low level of cytochrome a_3; therefore, we attribute the dominant Soret feature to cytochrome b_{595}.

No soluble *c*-type cytochromes were detected following heme staining of the soluble fractions of mutants, whereas in the soluble fraction of wild-type cells, a component with an M_r of 14,000 was present (Fig. 4a). This component was previously shown (37) to be from the periplasmic fraction. Two components (estimated M_rs of 31,000 and 23,000; Fig. 4b) were absent from the membrane fractions of the mutants compared with that of the wild type. These two components may be similar to two membrane-bound *c*-type cytochromes identified in *B. japonicum* (36). In *B. japonicum*, cytochrome c_1 was identified as a protein with an M_r of 28,000, and mutations of the *R. leguminosarum* gene encoding cytochrome c_1 abolish the formation of the component with an M_r of 31,000 (unpublished observations), confirming that it is cytochrome c_1.

Identification of the *cycHJKL* genes. Mutants A266 and A267 were complemented to NADI$^+$ by pIJ1942, a cosmid carrying 30 kb of DNA from *R. leguminosarum*. pIJ7076 containing a 10-kb *Hin*dIII fragment from pIJ1942 cloned in pML123 complemented the NADI$^-$ phenotype of both mutants. Cloning *Sac*I fragments from pIJ1942 into pML123 gave rise to pIJ1978, which carries a 3.3-kb insert that complements A267 to NADI$^+$; DNA hybridizations confirmed that this corresponded to the *Sac*I fragment into which Tn*5* had been inserted in A267. With A266 as the recipient, no complementing plasmid containing a single *Sac*I fragment was found, but pIJ7091 (which carries a 5.0-kb *Bam*HI fragment from the pIJ1942 insert) complemented A266 to NADI$^+$ (Fig. 1). DNA hybridization confirmed that the DNA cloned in pIJ7091 corresponds to the fragment into which Tn*5* is inserted in A266. Restriction enzyme mapping and DNA hybridizations revealed that pIJ1978 and pIJ7091 overlap by 1.4 kb (Fig. 1).

The DNA sequence of an ≈5-kb region spanned by pIJ1978 and pIJ7091 was determined, revealing four long ORFs (Fig. 1); the predicted protein sequences are shown in Fig. 5. All of

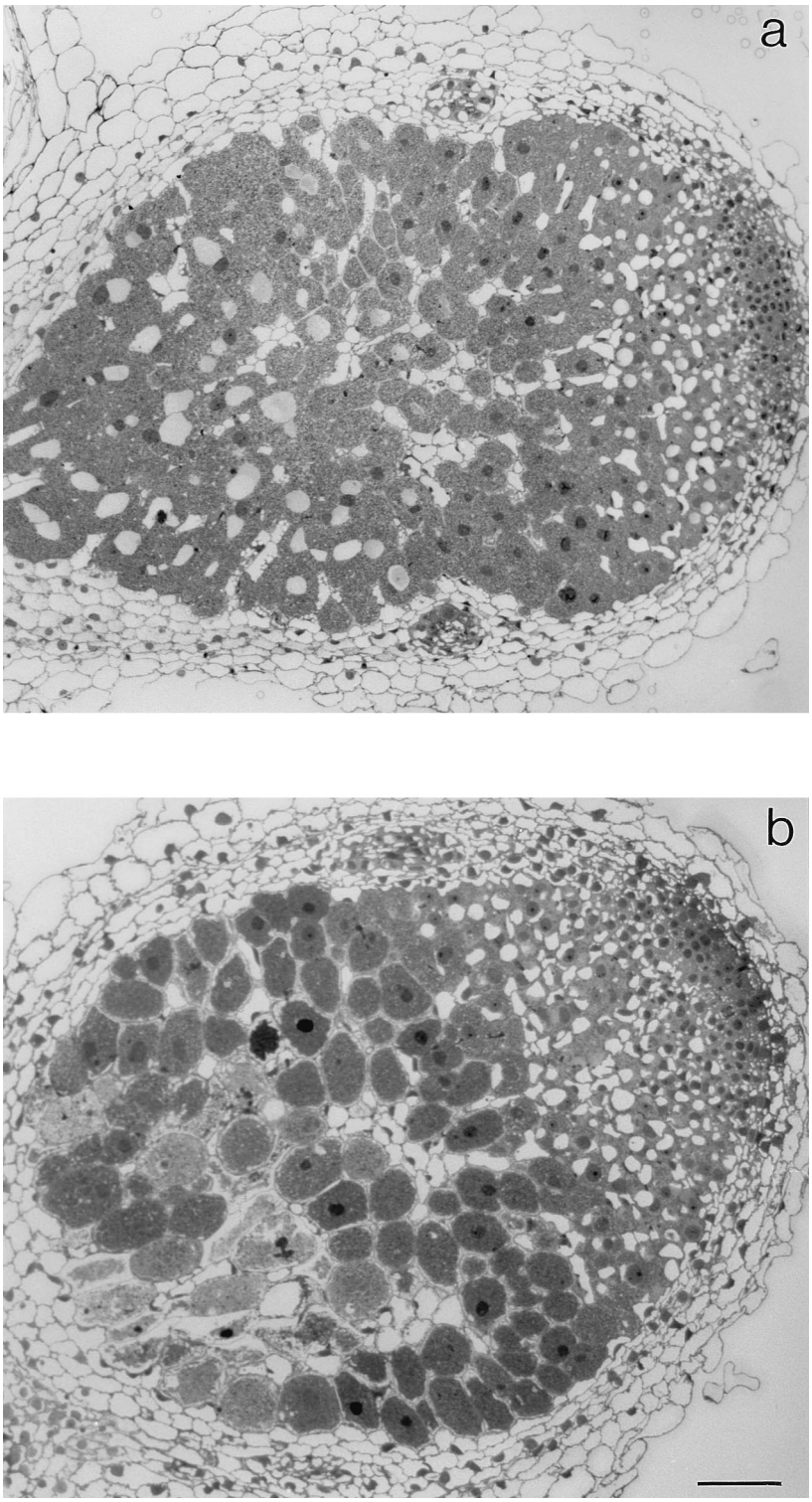

FIG. 2. Light micrographs of nodules. Nodules induced on peas by A33 (the wild-type control) (a) and the mutant A266 (b) were embedded and stained for light microscopy as described previously (5). Longitudinal sections of these nodules revealed that normal infection had occurred with A266 (*cycK67*::Tn*5*), although at the base of the nodule some premature senescence of infected cells can be seen. In addition, starch granules are evident around the edges of most of the infected cells in the nodule induced by A266. Similar results were observed with nodules induced by mutant A267 (*cycH271*::Tn*5*). Bar, 0.1 mm.

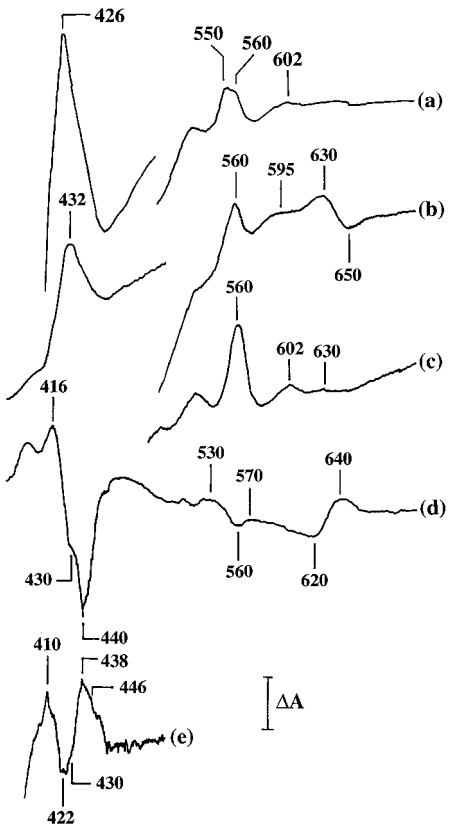

FIG. 3. Difference spectra of mutant A266 (*cycK67*::Tn*5*). Dithionite-reduced minus persulfate-oxidized difference spectra (recorded at room temperature) of whole cells from wild-type A33 grown in mannitol medium (a) and mutant A266 grown with mannitol (b) or succinate (c) as the carbon source; (d) CO-reduced minus reduced difference spectrum at room temperature; (e) photodissociation spectrum recorded at −100°C. The absorbance scale represents optical densities of 0.04 (a and c), 0.04 in the α region and 0.2 in the Soret region (b), 0.02 (d), and 0.004 (e). The protein concentrations were 7.15 mg ml^{-1} (a, b, d, and e) and 10.2 mg ml^{-1} (c), and the pathlength was 10 (a, b, c, and d) or 2 mm (e). Essentially identical spectra were observed with A267 (*cycH271*::Tn*5*).

the proposed translation start codons of ORFs 2, 3, and 4 overlapped with the termination codon of the upstream gene. Thus, the sequence at the junction of the first two ORFs is CAATGA; between ORFs 2 and 3, the sequence is CCATGA; and between ORFs 3 and 4, the sequence is GGATGA. In each case, the TGA encodes the predicted translation stop and ATG encodes the predicted translation start. This strongly suggests that the four genes are translationally coupled and hence in the same operon.

To locate the two Tn*5* mutations relative to these ORFs, flanking sequences were determined. The insertions from A267 and A266 lie in the first and third ORFs (Fig. 1), 87 and 915 nucleotides, respectively, from the proposed translation starts (Fig. 5).

The first ORF is predicted to encode a protein of 381 amino acid residues, with two potential membrane-spanning hydrophobic domains (residues 1 to 23 and 95 to 113; underlined in Fig. 5). The other regions of the protein are relatively hydrophillic. In database searches, similarities were seen with the *cycH* gene products from *B. japonicum* (40% identity), *R. meliloti* (57% identity), and *Paracoccus denitrificans* (30% identity). All of these *cycH* genes are involved in the assembly of *c*-type cytochromes (12, 19, 26). This is in keeping with our observations that mutations of the homologous gene block the formation of *c*-type cytochromes in *R. leguminosarum*. Accordingly, this gene was called *cycH* and the mutation in strain A267 was called *cycH271*::Tn*5*. There was also a lower (but significant) level of similarity to the *E. coli* gene, *nrfG* (22% identity), involved in the formation of a *c*-type cytochrome involved in formate-dependent nitrite reduction during anaerobic growth (11). Alignments of the *B. japonicum*, *R. meliloti*, and *P. denitrificans cycH* gene products with the *E. coli* NrfG protein have been presented recently (12, 19), also revealing similarity to the C-terminal region of YejP (20% identity), a predicted gene product with an undefined role in *E. coli* (25). Interestingly, DIAGON (33) analysis of the *R. leguminosarum* CycH against itself showed significant similarity between two domains of the protein, around residues 140 to 177 and 315 to 351 (conserved identities are marked with dots in Fig. 5, and the alignment is shown in Fig. 6). Similar repeated domains are also conserved in the same region of CycH proteins from *B. japonicum* and *R. meliloti* and may be weakly conserved in CycH from *P. denitrificans* (Fig. 6).

The second ORF (Fig. 5b) encodes a predicted protein of 165 amino acids. The N-terminal domain has four positively charged residues followed by a hydrophobic domain, characteristic of an N-terminal transit peptide to translocate the protein into the periplasm. Homologies (50 and 70% identity, respectively) were found with the *B. japonicum* and *R. meliloti cycJ* gene products, predicted to be periplasmic proteins involved in the assembly of *c*-type cytochromes (12, 27). Database searching also identified a similar predicted protein, YejS, from *E. coli* (35% identity). Alignments of these *B. japonicum*, *R. meliloti*, and *E. coli* proteins have been presented recently (12, 27).

The third ORF (Fig. 5c) encodes a predicted protein of 664 amino acids that is very hydrophobic and contains several potential transmembrane-spanning domains. It contains the putative heme-binding motif WGGWWFWD (35) at positions 237 to 244. The protein shows similarity (54% identity) to the *Rhodobacter capsulatus* Ccl1 proteins involved in the biogenesis of *c*-type cytochromes, and database searching also revealed homologies to similar proteins (called CycK) from *R. meliloti* (74% identity) and *B. japonicum* (63% identity) that are involved in cytochrome *c* biogenesis (12, 27). Other similar proteins are YejR (25) (45% identity) and NrfE (11) (38% identity) from *E. coli* and several mitochondrial and chloroplast

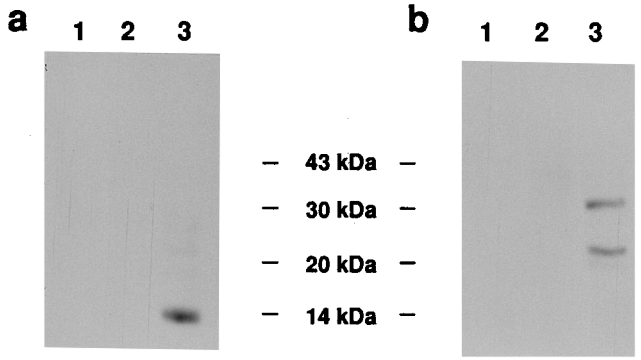

FIG. 4. Heme stains of soluble (a) and membrane (b) proteins. Soluble and membrane proteins from A267 (lane 1), A266 (lane 2), and the control (A33; lane 3) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and stained for heme proteins (37). Whereas a soluble protein (M_r, 14,000) and membrane components (M_rs, 31,000 and 23,000) were stained in the control, no significant staining was obtained with mutants. Each lane contained 50 (a) or 15 (b) μg of protein. The positions of molecular mass markers are indicated.

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(a) CycH
MLFWILVAALTAALAVILVYPLLRGQGPADNIRAGEAAVYRDQLRELDRD   50

LDGGLITPEEADYARAEIGRALIAVSADEPAETPKPARHHRFTEAFVLLV   100

LPVLGLCLYLTTGRPDLPSQPLEARLENPGNDVAVLITKAERHLAEKPDD   150

GKGWDVLAPIYFRTMRVDDAQVAYRNAIRLLGPSPVRLDGLAETLMAVSD   200

GVVTEEARQVLEQSVTLQPDNPRARFYIALSMEQAGQPDEARQAFEALAK   250

QSPSDAPWLPLVNQHIAMNGGAPAGQIRLHQALPAIPTQQDVAAAENMSA   300

GDRQQMIRGMVESLDAKLSEDPNNFEGWVRLVRSYAVLNDKDRAAGALKR   350

GLAAFPLACEEGRQLLALARELGIATEGATQ                      381
(b) CycJ
MTRNRKRLAVIAGGMGFILTAVLLVMFAFSQSVAYFYMPADLAKTPVAPE   50

TRIRLGGLVGEGSVVRGTGSTVEFAVTDGSTNPVKVKYTGILPDLFRGGQ   100

GVVTEDMFAAGTNVFVADTVLAKHDETYMPKDVADRLKSQGLWKEGQGQE   150

AQGKQAQGQEVKATP                                      165
(c) CycK
MIIEIGHYALVLALATALILSIVPVIGARRHDRAMMDVATIGSLAMFSLV   50

AFSFGVLTYAHVVSDFSVENVWENSHSLVPLLYKYSGVWGNHEGSMMLWL   100

LILTLFSALVAVFGRNLPETLKANVLSVQAWISVAFTLFILLTSNPFLRL   150

DPAPAEGRDLNPVLQDVGLAIHPPLLYLGYCRLLRCFSFAVAALLEGRID   200

AAWARWVRPWTLAAWTFLTLGIAMGSYSYWAYYELGWGGWWFWDPVENAS   250

FMPWLAGTALLHSALVMEKREALKIWTVLLAILTFSLSLMGTFLVRSGVL   300

TSVHAFASDPSRGVFILCILLIFIGGALSLFAFRAPRLSAGGLFAPISRE   350

GALVVNNPDLTVACGTVLTGTLYPLLLETLTGDKISVGPPFFNLTFGLLM   400

APLIVIVPFGPMLAWKRGDLLGALQRLYVVAGLAFLAAVIFFYIEHGGPV   450

LSVLGLAPGCSWILGAVADLWYRAGICIGRRAVLPGAGFPACRVRFGTAL   500

AHAGLGVTVLGIVAVTTFQSEHVIEMKPGEVTEAGGYSLHFDGMQPGTEP   550

NYTEERGHFTIRRAGVAVADTWSAKRLYTARQMPTTEAGILTFGLRQLYV   600

SLGDATKDGGIVVRIWWKPFILCIWGGAVFMAFGGLVSLSDRRLRVGAPA   650

KASAKPAPAMEPAE                                       664
(d) CycL
MMRRLLLAFALLLMAAPAFAVNPDEVLADPALETRARALSAELRCMVCQN   50

QSIDDSNADLAKDLRLLVRERITDGDSDEAVLNYIVSRYGEFVLLKPRVG   100

MKTVLLWGAPVLLVLAGGLSLLVFARKRAGKPTGSKLTADEQARLSELLK   150

K                                                    151
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FIG. 5. Predicted amino acid sequences of the *cycHJKL* gene products. The predicted amino acid sequences of CycH (a), CycJ (b), CycK (c), and CycL (d) are shown. The positions of Tn*5* insertions relative to these sequences are marked with triangles. Proposed N-terminal transit peptides are double underlined (b and d). Two proposed transmembrane domains in panel a are single underlined. Several potential transmembrane domains in panel c are not marked. Putative heme-binding sites (35) in CycK and CycL are in boldface. The dots above residues in panel a indicate identical amino acids conserved in the repeated domains found in CycH.

proteins also thought to be involved in cytochrome *c* biogenesis (30). The mutation identified in mutant A266 is located within this gene. According to the nomenclature used with *B. japonicum* and *R. meliloti*, we have called this gene *cycK* and the mutation *cycK67*::Tn*5*.

The fourth ORF encodes a protein of 151 amino acids which contains the potential heme-binding motif RCMVCQ (35) at residues 44 to 49 (Fig. 5d). There is a consensus N-terminal transit peptide, which probably translocates it to the periplasm, and indeed the predicted protein sequence is similar (54% identity) to the *Rhodobacter capsulatus* Ccl2 protein, which is also thought to be periplasmic (3) and is involved in cytochrome *c* biogenesis. Database searching also revealed homologies with the *cycL* gene products (12, 27) from *B. japonicum* (61% identity) and *R. meliloti* (68% identity); according to rhizobial gene nomenclature, we propose to call this gene *cycL*. There were also similarities with the *E. coli nrfF* gene product (31% identity) involved in the assembly of cytochrome c_{552} (related to nitrite reduction) and with the N-terminal domain of the proposed *E. coli yejP* gene product (38% identity), which interestingly was similar to CycH in the C-terminal domain.

DISCUSSION

c-type cytochromes are essential for symbiotic nitrogen fixation by rhizobia; we have defined a cluster of four genes (*cycHJKL*) involved in the biogenesis of cytochrome *c*. The observation that the proposed translation termination and initiation codons overlap suggests translational coupling and therefore cotranscription. Similar *cycHJKL* operons have recently been identified in *B. japonicum* (27) and *R. meliloti* (12). However, although genes (*ccl1* and *ccl2*) similar to *cycK* and *cycL* have been identified in *Rhodobacter capsulatus*, the genes upstream of this *ccl* operon are not involved in cytochrome *c* biogenesis (3). Presumably, homologs of *cycH* and *cycJ* are present in *Rhodobacter capsulatus* elsewhere in the genome. In *R. leguminosarum*, *cycH* is preceded by another gene (transcribed in the same orientation) that encodes a product with a sequence typical of membrane-bound lipoproteins (unpublished observations). A similar gene appears to be present at the same relative location in *R. meliloti* (12) but not in *B. japonicum* (27). Immediately upstream of this lipoprotein gene in *R. leguminosarum* are two other genes which appear to encode proteins homologous to a bacterial two-component transcriptional sensor regulator system (34, 38). The functions of these three upstream genes remain to be established, and their relationships, if any, to the nearby *cyc* genes have yet to be determined.

The observation that pIJ1978 can complement the *cycH271*::

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S. cerev.      P G Q K M D L P V D R T I S S I P K S P D S N E F W E Y P S P Q Q M Y N A M V R K G K I G G S G E   115
R. leg.        P G N D V A V L I T K A E R H L A E K P D D G K G W D V L A P I Y F R T M R V D D A Q V A Y R N A   177
R. mel.        P G D D V N I L I A K A E N H L A L N P Q D G A G W D L L A P I Y M R H G R L D D A V A A Y D R A   180
B. jap.        M A G S L E N L V V Q V E Q H L E K N P T D G R G W N V L A P V L E R L G R F D D A V R A Y R N S   180
P. denit.      S P C R I C R P D P S A A E A V Q K N P N D P R G L E L L A E H E E R L G N L V A A K D A Q H R L   200
E. coli NrfG   S Q Q T P E A Q L Q A L Q D K I R A N P Q N S E Q W A L L G E Y Y L W Q N D Y S N S L L A Y R Q A    99
E. coli YejP   N E E E M S R L A L G M R T Q L Q K N P G D I E G W I M L G R V G M A L G N A S I A T D A Y A T A   248
R. leg.        R Q Q M I R G M V E S L D A K L S E D P N N F E G W V R L V R S Y A V L N D K D R A A G A L K R G   351
R. mel.        R Q A M I R G M V D S L A S R L K E D P A N L E G W I R L V R S Y V V L D Q R D R A K D A L H D G   349
B. jap.        R G A M V R G M V E R L A T R L K Q N S D V E G W L R L V R A Y L V M G D R D K A V G A S S D A   344
P. denit.      R Q E M V E G M V K G L E T R L A T Q D G T A E E W A R L I S S L P V I G Q K D H A A D I L A E A   389

Consensus                                  L   N/DP  D/N   G W    L     Y/L            A    A
```

FIG. 6. Sequence comparisons of parts of the deduced amino acid sequence of *R. leguminosarum* CycH with those of other proteins. Two domains of CycH from *R. leguminosarum* (*R. leg.*; residues 129 to 177 and 303 to 351), *R. meliloti* (*R. mel.*; residues 132 to 180 and 301 to 349), *B. japonicum* (*B. jap.*; residues 132 to 180 and 296 to 344), and *P. denitrificans* (*P. denit.*; residues 152 to 200 and 341 to 389) are aligned to show the extent of the duplicated region in each protein. For comparison, parts of the *E. coli* NrfG (residues 51 to 99) and YejP (residues 200 to 248) proteins are included in this alignment. Boxes highlight highly conserved residues. The region of *R. leguminosarum* CycH extending from residues 129 to 160 showed some similarity with residues 67 to 100 of the *Saccharomyves cerevisiae* (*S. cerev.*) cytochrome *c* lyase by DIAGON analysis, and identical residues contributing to this similarity are indicated by dots.

Tn*5* mutation indicates that this mutation is not fully polar on the downstream genes, *cycK* and *cycL*, which are not present on pIJ1978. This implies that a promoter within Tn*5* allows expression of the downstream genes or that there is a promoter within the coding sequence of *cycH* or *cycJ*. We favor the former hypothesis, since there are other examples of Tn*5* mutations in *Rhizobium* spp. that are not strongly polar (29).

Although we have formally proven only that *cycH* and *cycK* are essential for the biogenesis of *c*-type cytochromes, the strong conservation of *cycJ* and *cycL* relative to other bacterial genes involved in cytochrome *c* biogenesis indicates that the *cycJ* and *cycL* gene products also play a role in cytochrome *c* assembly. Significantly, a mutation of *cycH* or *cycK* blocks the formation of several *R. leguminosarum* *c*-type cytochromes, resulting in a significant decrease in A_{550}. Heme staining revealed two *c*-type cytochromes that are associated with membrane fractions of free-living cultures of *R. leguminosarum*; these components were absent from strains with mutations in *cycH* or *cycK*. In addition, the formation of a periplasmic *c*-type cytochrome was blocked by these mutations.

The final stage in assembly is the attachment of heme to apocytochromes *c*; this appears to occur in the periplasm (35). The proposed locations of two gene products (CycJ and CycL) in the periplasm, the presence of potential heme-binding domains in CycK and CycL, and the proposed transmembrane locations of CycH and CycK are compatible with such a model. Following the model of CycH proposed previously (26), the C-terminal region of about 220 amino acid residues is likely to be in the periplasm. The presence of a repeated domain in this proposed periplasmic region in the three rhizobial CycH proteins might indicate that there are two active sites in CycH. Repeated domains were not found in the *E. coli nrfG* and *yejP* predicted gene products, and only a weakly conserved repeat was found in CycH from *P. denitrificans* (Fig. 6). DIAGON (33) comparison of the *R. leguminosarum* CycH with yeast cytochrome *c* heme lyase (with relatively stringent parameters of a score of 324% and a window of 29) revealed a short region of similarity for these two proteins. This similarity was located around residues 67 to 100 of the yeast heme lyase and around residues 129 to 160 of CycH (identities are marked in Fig. 6), indicating that this region may be involved in heme attachment.

Although cytochrome c_1 is necessary for electron transport during symbiotic nitrogen fixation (36), there is no evidence to suggest that the other *c*-type cytochromes detectable in *R. leguminosarum* during free-living growth are necessary for symbiotic nitrogen fixation. However, it appears that specific *c*-type cytochromes (encoded by *fixO* and *fixP*) are induced in bacteroids (16, 22) and that these are essential for symbiotic nitrogen fixation. Therefore, the *cycHJKL* gene products might be involved in the attachment of heme to FixO and/or FixP apoproteins, as well as cytochrome c_1.

The stage of nodule development reached by the *cycH* and *cycK* mutants of *R. leguminosarum* is markedly different from that observed in nodules induced by *B. japonicum* and *Rhizobium phaseoli* mutants lacking *c*-type cytochromes (26, 32). In the pea nodules induced by *R. leguminosarum* bv. *viciae* mutants, cells are fully infected with many bacteroid forms surrounded by peribacteroid membranes. In contrast, soybean nodules induced by *cycH* mutants of *B. japonicum* contained very few bacteroids (26). In fact, the DNA sequence of a gene previously thought to be specifically involved in bacteroid release (18) shows near identity with the *cycH* gene (26; also our observations). These differences in nodule development could reflect differences in the characteristics of the bacteria or the legume host. *R. phaseoli* is much more similar to *R. leguminosarum* than *B. japonicum* is, but the nodules induced on *Phaseolus* beans by a cytochrome *c*-deficient mutant of *R. phaseoli* lack bacteroids (32). The nodules formed on soybeans and *Phaseolus* beans have a determinate meristem distinct from the indeterminate meristem of pea nodules. Therefore, the lack of significant infection of nodule cells by cytochrome *c*-deficient rhizobia may be a function of the type of nodules formed.

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ADDENDUM IN PROOF

After submission of this paper, we were informed of a paper by L. Thöny-Meyer, F. Fischer, P. Künzler, D. Ritz, and H. Hennecke (J. Bacteriol. **177:**4321–4326, 1995) in the August issue. In that work, the *yej* genes in the *E. coli aeg*-46.5 cluster were shown to be required for cytochrome *c* biosynthesis. It was proposed that these genes referred to here as *yej* be renamed *ccm* (cytochrome *c* maturation), altering *yejP* to *ccmH*, *yejR* to *ccmF*, and *yejS* to *ccmE*.

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